Master's Thesis of Science in Agriculture

Overexpression of *OsTZF8*, a CCCH-Tandem Zinc finger, increases drought tolerance in rice

CCCH –Tandem Zinc finger인

***OsTZF8*의 과발현 벼의 가뭄저항성 증가에 대한 연구**

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ABSTRACT

Overexpression of *OsTZF8*, a CCCH-Tandem Zinc finger, increase drought tolerance in rice

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Tandem CCCH zinc finger proteins (TZFs), members of the zinc finger protein family, have been known to participate as post-transcriptional regulators of gene expression in eukaryotes. However, the function of TZF as a drought stress-related gene remains unclear. Here, we show that the *OsTZF8*, a rice gene for tandem CCCH zinc finger protein, is induced by abiotic stress and its transgenic rice plants overexpressing *OsTZF8* increases drought tolerance. Gene expression analysis revealed that *OsTZF8* is specifically expressed in embryo, but not in vegetative organs. In field evaluation, grain yield and seed size were higher in the transgenic plants with constitutive overexpression than non-transgenic plants under drought conditions. *OsTZF8* not only localizes in the nucleus, but also co-localizes with both processing bodies and stress granules, two messenger ribo-nucleoprotein complexes which are known to activate by forming cytoplasmic foci under stress conditions. Taken together, these results suggest that *OsTZF8* participates in RNA turnover in processing bodies and stress granules regulating drought stress in rice.

Keywords

Tandem CCCH zinc finger, Rice, Drought tolerance, Processing bodies (PB), Stress granules (SG)

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CONTESTS

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LIST OF ABBREVIATIONS

35s	*Cauliflower mosaic virus 35S promoter*
ABA	Abscisic acid
SZF1	salt inducible zinc finger
CCCH	Cysteine3Histidine
cDNA	Complementary DNA
GFP	Green Fluorescence Protein
LEA	Late Embryogenesis Abundant
NT	Non-transgenic
PB	Processing bodies
PCR	Polymerase Chain Reaction
PGD1	*Phosphogluconate dehydrogenase 1*
qRT-PCR	Quantitative Real-Time PCR
RNA	Ribonucleic Acid
SG	Stress Granules
TZF	Tandem Zinc Finger
Wsi18	*Water-stress inducible protein 18*

INTRODUCTION

World's food demand is a one of the most critical issues currently. Not only has this demand originated from sharply increasing world population, but also from dramatic climate change happening worldwide. Noticeable change is simultaneous temperature increase globally, causing severe drought. It is expanding at rapid speed in every country, including Korea, which has become already one of the countries that should worry about drought. This year, especially, showed harsh amount of rainfall as it decreased almost 40% in average. This change shows proof that drought has become another key factor in crops, especially rice apart from nitrogen, which had been a major concern in Korea for quite some time. Many people agree that only doing continuous breeding of naturally born crop is insufficient to resolve this demand. In fact, conventional breeding methods have already been using agricultural technologies to improve current crops (Varshney et al. 2011). Plant biotechnology provides a powerful solution to boost agricultural productivity, to protect the environment and even to enhance nutritional quality. Since the first commercialization of the FLAVR SAVR tomato in 1994, the transgenic crops have been an economically important part of the world's crop industry (Aldemita et al. 2015; Kamthan et al. 2016).

Our gene, Tandem Zinc finger 8, whose family, Cysteine3Histidine (CCCH) zinc finger proteins are unfamiliar considering drought related. However, a microarray data we have proceeded previously have acknowledged the fact that *OsTZF8* (*OsC3H10*) is induced by drought stress (Oh *et al*., 2009). *OsTZF8* are characterized

by a zinc finger motif consisting of three cysteines and one histidine coordinated by a zinc cation. Genomic analyses have revealed 68 and 67 CCCH zinc finger protein genes in Arabidopsis and rice, respectively (Wang et al. 2008). The majority of Arabidopsis CCCH zinc finger proteins contain one or two zinc finger motifs. In the latter group, 11 members containing a plant-unique tandem CCCH zinc finger (TZF) motif preceded by an arginine-rich (RR) region (Pomeranz et al. 2010a). The functions of most of the genes in this unique subfamily are unknown, but recently, a few genes of Arabidopsis, *PEI1* (an embryo-specific zinc finger protein gene required for heart-stage embryo formation in Arabidopsis), *AtSZF1/AtSZF2* (for the salt inducible zinc finger of Arabidopsis), and *SOMNUS*, were characterized to function in embryogenesis (Li and Thomas, 1998), salt stress response (Sun et al., 2007), and light-dependent seed germination (Kim et al., 2008), respectively. Through this study, we confirm that there is a role of *OsTZF8* gene in the plant defense system as the drought tolerance gene in Oryza sativa (rice), possibly through the control of RNA metabolism of stress-responsive genes

MATERIALS AND METHODS

1. Plant growth for abiotic stress and stress treatment

Non transgenic rice seeds (illmi) were planted on solid Ms0 medium, 20 seeds each. After germinating in 28℃ dark growth chamber for 3 days and light chamber for 1 day, plants were transplanted in 7X14 pot, 2 in each pot. Sampling was performed 2 weeks after transplant. Before sampling, plant was removed from the soil and put in the dH_2O with room temperature for 72 hours to reduce other stresses. The root should not receive light during these 72 hours as root formation can alter. After 72 hours, 15 plants were grouped for each abiotic stress treatment. Drought stress was performed by air drying the whole plant on the tray, placed under indirect sunlight to prevent too much harsh drought stress. 400mM of NaCl was dissolved into water for salinity stress and 100μl of ABA was dissolved in 50% of EtOH for ABA stress. Cold stress was induced by placing the plant in 4℃ condition. After 2, 4, 6 hours incubation, sampling was done and immediately was frozen in liquid nitrogen to decrease the risk of RNA destruction.

2. Plant growth for visual test and drought stress tolerance test

Non transgenic rice seeds and each line with PGD1 promoter and RCc3 promoter were planted on solid Ms0 medium, 20 seeds in each plate. After germinating in 28℃ dark growth chamber for 3 days and light chamber for 1 day, each line was transplanted to 5X10 pot, 3 in each pot, and 30 plants in total. The

plants were grown for 4 weeks in the greenhouse after transplant and then removed from water. The observation continued for 3-4 days until rolling of leaves of plants is seen. Each symptom was recorded by using a NEX-5N camera (Sony, Japan). Soil moisture was measured by SM150 machine.

3. RNA extraction

After blending the leaf and root, TRIzol Reagent was used to extract RNA always starting with 1ml of TRIzol Reagent in order to maximize change of extracting plenty amount of RNA from the sample. First homogenize the samples by 1ml of TRIzol Reagent. Following homogenization, centrifuge the sample at 12,000 × g for 10 minutes at 4℃ is done, which would isolate RNA at supernatant apart from tissue or cells with high content of fat, protein, polysaccharide, or extracellular material. To continue to phase separation the aqueous phase is placed in a new tube and then incubate the homogenized sample for 5 minutes at room temperature is necessary to permit the complete dissociation of the nucleoprotein complex. Then 0.2 ml of chloroform per 1 ml of TRIzol Reagent used for homogenization is added and shake the tube vigorously by hand for 15 seconds. Another incubation is done for 2–3 minutes at room temperature and centrifuge the sample at 12,000 × g for 15 minutes at 4℃. Removing the aqueous phase of the sample should be done very carefully avoiding drawing any of the interphase or organic layer into the pipette when removing the aqueous phase. The aqueous phase is placed into a new tube to do RNA precipitation. 0.5 ml of 100% isopropanol to the aqueous phase, per 1 ml of TRIzol Reagent used for homogenization is added and incubated at room temperature for 10 minutes and centrifuged at 12,000 × g for 10 minutes at 4℃. The

next step is RNA wash. This time the supernatant from the tube is removed, leaving only the RNA pellet. By using 1 ml of 75% ethanol per 1 ml of TRIzol Reagent, washing the pellet is done. Slight vortexing is necessary for proper washing of the sample, then centrifuge the tube at 7500 × g for 5 minutes at 4℃ and discard the ethanol. The tube should be completely dry before elusion as ethanol affects 260/280 ratio when checking the purity of the RNA. The last process is elution used RNAse free water.

4. cDNA synthesis

As RNA is very unstable continuing at least to cDNA synthesis is recommended for better RT-PCR result. Before cDNA synthesis the concentration of each RNA should be set into same dose using NANO DROP. The volume should be calculated to 11.5 µl. First Oligo primer 1 µl is added and incubated at 70°C for 5min, then chilled on ice (4℃). The master mix should be made in this order 1.5x reaction buffer 4µl, 2. RiboLock™ Ribonuclease Inhibitor (20u/µl) 0.5µl, 3. 10mM dNTP mix 2µl 4. Reverse transcriptase (20u/µl) 2µl for the final volume 20µl. The mixture is incubated at 42℃ for 90min and the reaction is stopped by heating at 70℃ for 10min then chill on ice. For storage, cDNA is usually diluted 20% by adding 80µl of dH_2O.

5. Quantitative real-time PCR

For quantitative real-time PCR (qRT-PCR) experiments, total RNA was extracted from leaves of 30-day-old transgenic and non-transgenic (NT) rice plant by using the GeneAll RiboExTM LS Kit (GeneAll Biotech, Korea) and followed

cDNA synthesis by RevertAid First-Strand cDNA Synthesis Kit (Fermentas). qRT-PCR was carried out using a Platinum. Rice Ubiquitin1 (Os06g0681400) transcript abundance was used as the normalizing internal control, and three biological replicates were analyzed for all quantitative experiments. The *OsTZF8* specific primers used for qRT-PCR are 5'-CATGAAGCAGATTGTCCTTGC -3' and 5'-TCAACAGGTCAGACACCCA -3'.

6. **Agronomic traits analysis**

To evaluate the yield components of transgenic rice plants under normal field condition, 4 lines of T5 homozygous transgenic rice and NT plants were planted in a rice paddy field at the Kyungbook National University, Gunwi (128:34E/36:15N), Korea. A randomized design was employed for three replicates using 3 different 10 m2 plots. Approximately, 30 plants per line were sown and 6 seedlings per line were transplanted into the plots at 25 days after sowing. Fertilizer was applied at 70N/40P/ 70 K kg/ha after the last paddling. Yield parameters were scored with the 30 plants per line collected from three different plots for normal field condition. The results from independent lines were compared with those of NT controls, using analysis by one-way ANOVA. The detailed information for the agronomic trait analysis was described by Lee et al. (2016).

7. **Subcellular localization using rice protoplast**

Protoplast isolation was done from 10 days old rice plant (*O. Sativa* cv. Dongjin). The method was followed by previously described Jung et al., (2015). After 12 hours incubation at 28℃, the protoplast was harvested and measured using

Leica SP8 STED laser scanning confocal microscope (Leica, Germany).

RESULTS

1. Phylogenetic tree of Zinc finger in the Oryza sativa

The plant has a unique, characterized TZF motif compare to mammalian TZF protein. Most of the mammalian TZF proteins are characterized by a TZF motif with two identical C-x8-C-x5-C-x3-H zinc fingers in tandem, separated by 18 amino acids (Blackshear and Lai 2005). On the other hand, Arabidopsis and rice RR-TZFs contain a unique TZF motif of C-x7–8-C-x5-C-x3-H-x16-C-x5-C-x4-C-x3-H, except for *OsTZF7, OsTZF8* and *OsTZF9* that are characterized by a variant motif of C-x10-C-x5-C-x3-H-x16-C-x5-C-x4-C-x3-H and C-x15-C-x5-C-x3-H-x16-C-x5-C-x4-C-x3-H, respectively (Wang et al. 2008). The phylogenetic tree of all 67 CCCH zinc finger in rice based on the protein sequence alignment shows that Tandem zinc fingers with unique TZF motif are branched close to each other (Fig1a). The alignment of protein sequence of all nine TZF family demonstrates the conserved TZF motif highlighted with blue and very similar patterns of other amino acids (Fig1b). The *OsTZF8* also contains C-x10-C-x5-C-x3-H-x16-C-x5-C-x4-C-x3-H and C-x15-C-x5-C-x3-H-x16-C-x5-C-x4-C-x3-H pattern of conserved TZF motif and arginine rich region, highlighted in red (Fig1c).

2. Expression patterns of *OsTZF8*

OsTZF8 were selected as drought induced gene through microarray experiment performed earlier (Oh *et al*., 2009). In order to check the pattern of expression in two separate tissues, 21 days old illmi plant was provided with four

abiotic stresses, drought, high salinity, abscisic acid and low temperature and sampled after 2,4,6 hours. At the shoot, expression of *OsTZF8* turned up to have an increasing pattern in both drought and high salinity as the specific stress exposure time increases. (Fig 2a). At root only abscisic acid demonstrated increasing pattern indicating that *OsTZF8* plays inconsistent role in shoot and root when different abiotic stress are present (Fig 2b).

In Rice xpro, there is almost no expression of *OsTZF8* in other development stage except in embryo. Therefore, the observation of expression level was separated with the early germination stage and the rest of developmental stage. The sampling of the overall developmental stage was done first from 3days after germination in medium grown in a dark chamber before moving into the light chamber until after heading stage. The meiosis leaf was chosen as a control as it had the highest ct value meaning lowest expression level among the stages whose ct value was confirmed during 40 cycles of qRT-PCR. Some of the stages such as before, heading leaf or before heading flag leaf, the CT value was not on site until 40 cycles of QRT-PCR. Therefore the expressions of these stages are considered to be lower than meiosis leaf.

There is no significant pattern in the expression level of the developmental stage (Fig 2c). There is an increase of expression in root at an early stage and at meiosis flower. It is a noticeable result yet, it cannot be linked to a reliable order. In early germination stage, the expression significantly drops after germination compared to the expression level in embryo stage (Fig 2d).

3. Drought stress tolerance visual test of *PGD1:OsTZF8* and *RCc3:OsTZF8*

In order to test their tolerance to drought stress, 5 weeks old transgenic and non-transgenic plants were withheld watering for 4d, then re-watered for 5d. Almost all the leaves of non-transgenic plants illustrated extremely rolled up figure at the third day after water cut while transgenic lines were visible to have practically no rolled up leaves (Fig 3a). All four lines showed high expression in leaf and root compared to non-transgenic plant from minimum 300 to 1000 (Fig 3b). The graph of soil moisture contents proves the continuous drop of water in soil of both transgenic and non-transgenic plants, although the water content in the soil of transgenic lines happen to decrease slower, which may indicate the less use of water in order to survive (Fig 3c). Checking the survived plants, which was calculated by considering plant which recovered to its state beforehand withheld watering after 5 d of re-watering as survived plant. The survival rate of transgenic lines was from 91.7% to 100%, significantly higher than those of non-transgenic plants which is 12.5% (Fig 3d).

The drought tolerance was also detected in *RCc3:OsTZF8* transgenic lines with root specific promoter yet not as strong as in *PGD1:OsTZF8*. Although all the transgenic lines exhibit both high expression level and strengthen drought tolerance than non-transgenic plant (Fig 3e, f), the survival rate fluctuated from 40% to 100% by different lines (Fig3 h).

4. Agronomic trait of Overexpression transgenic plant of *OsTZF8* and non-transgenic plant grown under normal and drought condition in the field

In order to evaluate the differences happening in other phenotypic characteristics, both *PGD1:OsTZF8* and *RCc3:OsTZF8* were grown with each cultivar non-transgenic plant under normal condition and drought condition in the field. The most of characteristics of *PGD1:OsTZF8* have decreased except 1000 seed weight indicating that the size of the seed has grown in normal condition (Fig 4 a). In drought condition, however, the size of the seed showed no significant change yet the filling rate and number of filling grain increased dramatically (Fig4 b). These two data implying that the *PGD1:OsTZF8* has strong drought tolerance and may be participating in the seed germination pathway. On the other hand, the *RCc3:OsTZF8* displayed no meaningful phenotype, except an increase in filling rate under drought condition.

5. Phenotypic characterization of grains in NT and PGD1:OsTZF8 transgenic plant

To double confirm the fact that the size of grain has increased in two lines #20 and #23 of *PGD1:OsTZF8*, a photo of the same number of grains (n=10) aligned to examine the size difference in length (Fig 5 a). We observed a substantial increase in grain length as almost one grain difference was monitored at 10 grain alignment. This difference was examined in both with and without bran (Fig 5 a, b). These results indicate that the increase in grain weight was because of an increase in grain length.

6. Subcellular localization of *OsTZF8*

When the first subcellular localization was performed using protoplast, a unique result was observed as *OsTZF8:GFP* was monitored not only at nucleus, but also in parts of cytoplasm, some forming a foci(data non shown). As there are studies that other family member of TZF was sighted at PB and SG (Jan et al., 2013), there was a chance that our gene works in the same direction. To confirm whether *OsTZF8* is associated with PBs and SGs, colocalization analysis using Arabidopsis marker genes for PBs and SGs was performed. *OsTZF8* was fused in frame with green fluorescent protein, and the marker genes were fused with cyan fluorescent protein. DCP1-2 is an mRNA-decapping enzyme and localizes to PBs (Iwasaki et al., 2007; Weber et al.,2008). Both constructs were introduced into protoplasts prepared from rice shoot cells.

To confirm that 35S promoter vector is working properly, *35S:GFP* was first observed as a control (Fig 6a). *OsTZF8:GFP* was primarily stained in dapi, in order to check the expression in the nucleus and the result shows that it does have activation in the nucleus (Fig 6b). Then *OsTZF8:GFP* colocalized with DCP1-2 in cytoplasmic foci resembling PBs (Fig. 6c, d). Colocalization of *OsTZF8* was also tested in SGs using the SG marker PABP8 (Newbury et al., 2006; Anderson and Kedersha, 2008). *OsTZF8* and PABP8 colocalized in comparatively large cytoplasmic foci, considered to be SGs (Fig. 6b). When heat stress was given, more foci was unleashed. Maximum of 3 hours of 42℃ of heat stress was given as longer heat stress happened to cause protoplast to dry and demolish. *OsTZF8:GFP* seems to form less foci in PB when heat stress is given than SG which show increased

number of foci after 3 hour stress given.

7. Candidates of *OsTZF8* target genes based on RNA-seq data

RNA-sequencing was done with 3 weeks grown non transgenic plant and *PGD1:OsTZF8* #20 with biological repeat as line #20 showed the best phenotype in the drought tolerance visual test. The candidate gene was selected first by comparing RNA sequencing data analysis on 1d, 2d, 3d drought treated samples (Chung *et al*., 2016). Then, the candidates were narrowed down to certain genes which is either related to plant defense or embryogenesis or both. Among 865 genes deleting genes that is currently stated as 'Unknown function (DUF)', 'Non-protein coding transcript' and 'Conserved Hypothetical protein' as although these genes could happen to be related to *OsTZF8*, at this state, it is difficult to figure out the role of these genes. They were collected into 3 groups, late embryogenesis abundant protein (LEA), Germin-like protein (GLP) and Pathogenesis related (PR). Total 22 genes are considered to be candidates of *OsTZF8* target gene (Table1).

Out of 22 genes 7 genes showed an increase in expression level in transgenic plant using QRT-PCR (Fig. 7). Os01g0348900 which is commonly known as salt stress inducible marker SalT1, 3 LEA genes, Os05g0542500, Os11g0454200, and Os03g0168100, and 3 Germin like genes Os08g0189400, Os08g0189400, Os12g0154800. Each expression level increased from minimum 1.7 times to maximum 14 times.

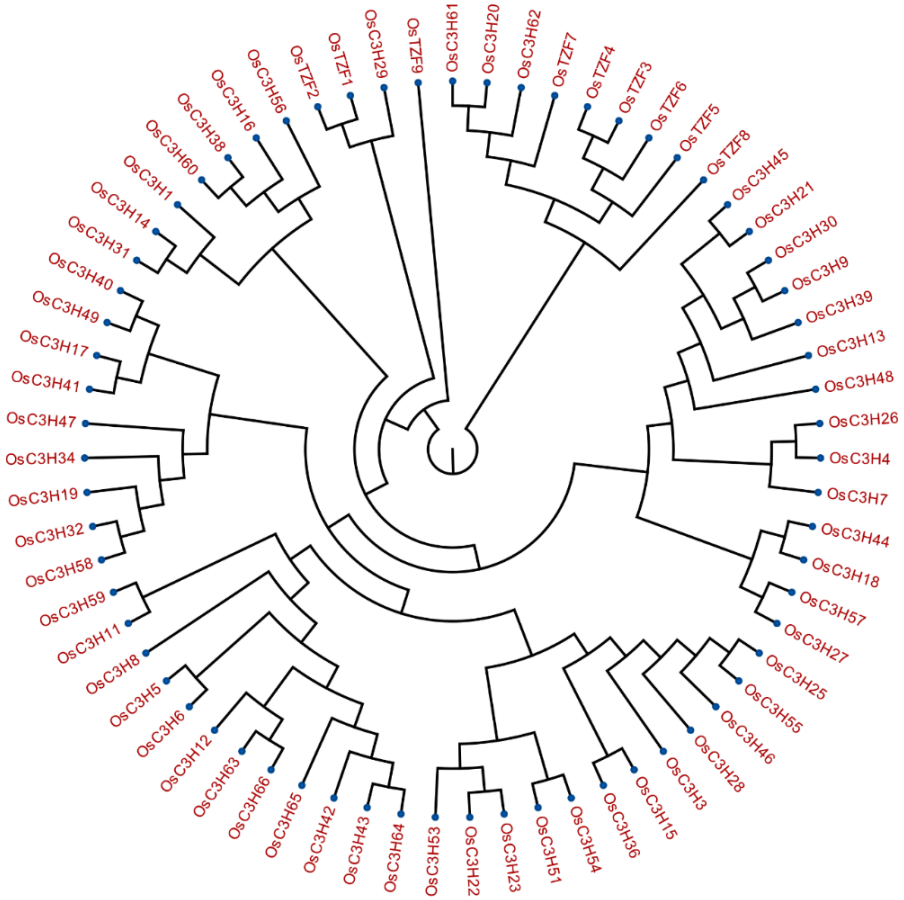
Fig. 1 Phylogenetic tree of Zinc finger in the Oryza sativa and Conserved TZF motif of TZF proteins.

a. Phylogenetic tree of 67 zinc finger in Oryza sativa made by CLC workbench software based on full-length amino acid sequences.

b. Protein alignment of the nine TZF zinc finger family member. Amino acid alignment was also performed with CLC sequence viewer. The blue highlighted part shows the conserved CCCH motif of nine TZF zinc finger proteins

c. Whole protein sequence of OsTZF8,. The blue highlighted part shows the conserved CCCH motif and red highlighted part show arginine rich region

a.


OsTZF9
OsC3H61
OsC3H20
OsC3H62
OsTZF7
OsTZF4
OsTZF3
OsTZF6
OsTZF5
OsTZF8
OsC3H45
OsC3H21
OsC3H30
OsC3H9
OsC3H39
OsC3H13
OsC3H48
OsC3H26
OsC3H4
OsC3H7
OsC3H44
OsC3H18
OsC3H57
OsC3H27
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OsC3H28
OsC3H3
OsC3H15
OsC3H36
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OsC3H34
OsC3H47
OsC3H41
OsC3H17
OsC3H49
OsC3H40
OsC3H31
OsC3H14
OsC3H1
OsC3H60
OsC3H38
OsC3H16
OsC3H56
OsTZF2
OsTZF1
OsC3H29

b.

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OsTZF1  EDEEEAAAVAAAVD----------AYACDEFRMYEFKVRRCARGRSHDWTECPFAHPGEKARRRDPRRYCY 180
OsTZF2  DEEEEAAAMAAAVD----------AYACDEFRMYEFKVRRCARGRSHDWTECPFAHPGEKARRRDPRKYHY 117
OsTZF3  SEKKEYPVDPSLPDI------KNSIYASDEFRMYSFKIRPCSRAYSHDWTECPFVHPGENARRRDPRKYHY 322
OsTZF4  --RKEWPPDPSLPDI------KNGAYASDDFRMYSFKVRACSRAYSHDWTECPFVHPGENARRRDPRKYHY 214
OsTZF5  EAKKEYPPDLTLPDL------KSGLPSTDEFRMYSFKVKPCSRAYSHDWTECPFVHPGENARRRDPRRYSY 253
OsTZF6  RGKKEYPVDPTLPDI------KSSVYASDEFRMFAFKVRPCSRAYSHDWTECPFVHPGENARRRDPRKHPY 275
OsTZF7  -DGEAEADAEAEAD-----------EADDEFMMYEFKVRRCARARSHDWTACPYAHPGEAARRRDPRRVAY 99
OsTZF8  -SSPTWG-GRCAYD-----------AYDEDFMMYEFKVRRCPRSRAHEWTSCPYAHPGEAARRRDPSHVTY 85
OsTZF9  HEEEEVTIDPTKWGAWAHRGHRLWASMSEDFWIHVYKVQRCPRSSSHDWTSCPYAHKGERARRRDTRRFAY 89

OsTZF1  SGTACPDFRK--GG------CKRGDACEFAHGVFECWLHPARYRTQPCKDGTACRRRVCFFAHTPDQLRVL 244
OsTZF2  SGTACPDFRK--GG------CKRGDACEYAHGVFECWLHPARYRTQPCKDGTACRRRVCFFAHTPDQLRVL 180
OsTZF3  SCVPCPDFRK--GV------CRRGDMCEYAHGVFECWLHPAQYRTRLCKDGTSCNRRVCFFAHTTDELR-- 383
OsTZF4  SCVPCPEFKK--GA-----GCRRGDMCEYAHGVFESWLHPAQYRTRLCKDGVGCARRVCFFAHTPDELR-- 276
OsTZF5  SCVPCPEFRK--GG-----SCRKGDACEYAHGVFECWLHPAQYRTRLCKDEVGCARRICFFAHKPDELRAV 317
OsTZF6  TAVPCPNFRR--PG-----GCPSGDSCEFSHGVFESWLHPSQYRTRLCKEGAACARRICFFAHDEDELR-- 335
OsTZF7  TGEPCPDFRRRPGA-----ACPRGSTCPFAHGTFELWLHPSRYRTRPCRAGVACRRRVCFFAHTAGELRA- 164
OsTZF8  TGEPCPDFRVAARA-----ACPRGSGCPFAHGTFETWLHPSRYRTRPCRSGMLCARPVCFFAHNDKELRI- 150
OsTZF9  AAVSCPDYRPREAAPGAVPSCAHGLRCRYAHGVFELWLHPSRFRTRMCSAGTRCPRRICFFAHSAAELRDP 161
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c.

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1   M A Y E T S S D H Q L A A A A E F L A A L Q V H L A G A E A
31  S S P T W G G R C A Y D E D F M M Y E F K V R R C P R S R A
61  H E W T S C P Y A H P G E A A R R R D P S H V T Y T G E P C
91  P D F R V A A R A A C P R G S G C P F A H G T F E T W L H P
121 S R Y R T R P C R S G M L C A R P V C F F A H N D K E L R I
151 V G D D A A A A T P S P R S P F T T S E D S P P P S P M D M
181 K Q I V L A M Q Q M D A R K A T R S V A P K T D M L Q Q E L
211 E E D A P E L G W V S D L L M
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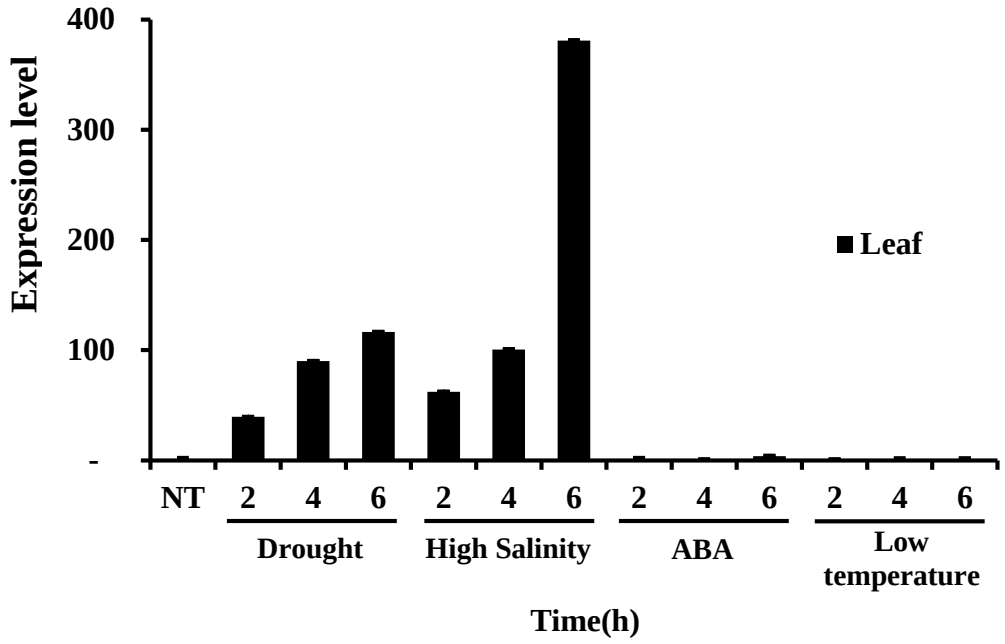
Fig. 2 Expression patterns of *OsTZF8* in response to four different abiotic stresses according to time of treatment in leaf (a), root (b), different developmental stage (c) and in early germination stage (d).

a,b. Non transgenic plant were planted in Ms medium, grown in dark for 4days, grown in light for 1day and transplanted to the soil and grown for another 2 weeks. The plants were then transferred to dH2O for 3 days adaptation. The plants were treated with four different abiotic stresses, high salinity (400mM), drought (air dry), ABA treatment (100μM), and low temperature (4℃).

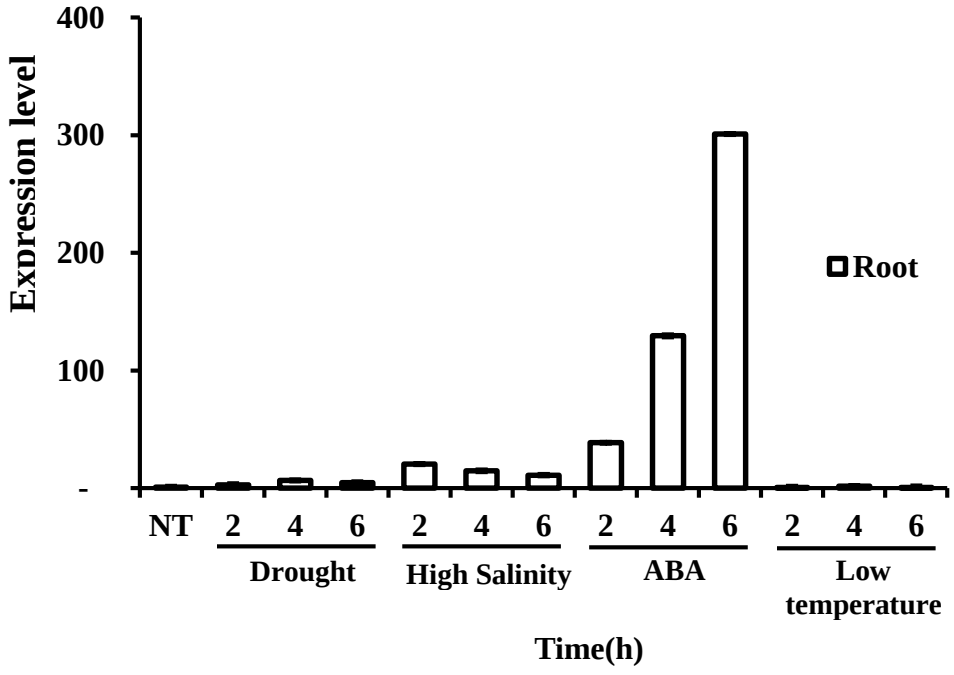
c. RNA collected at each time of development stage from 3 days after germination at medium in a dark chamber to after heading was used for quantitative RT-PCR for analysis. '1 month Leaf' is considered as control as it showed the lowest expression level. D, Dark, Front L, Light, Back L, Leaf, H, Heading, R, Root, c, cotyledon, S, Shoot, FL, Flag Leaf, F Flower

d. Early germination stage samples were collected from seed considered as 0 hours, and 12 hours, 24 hours, 48 hours after the seed was planted in Ms0 medium in a dark chamber. '48 hours' is considered as control as it showed the lowest expression level.

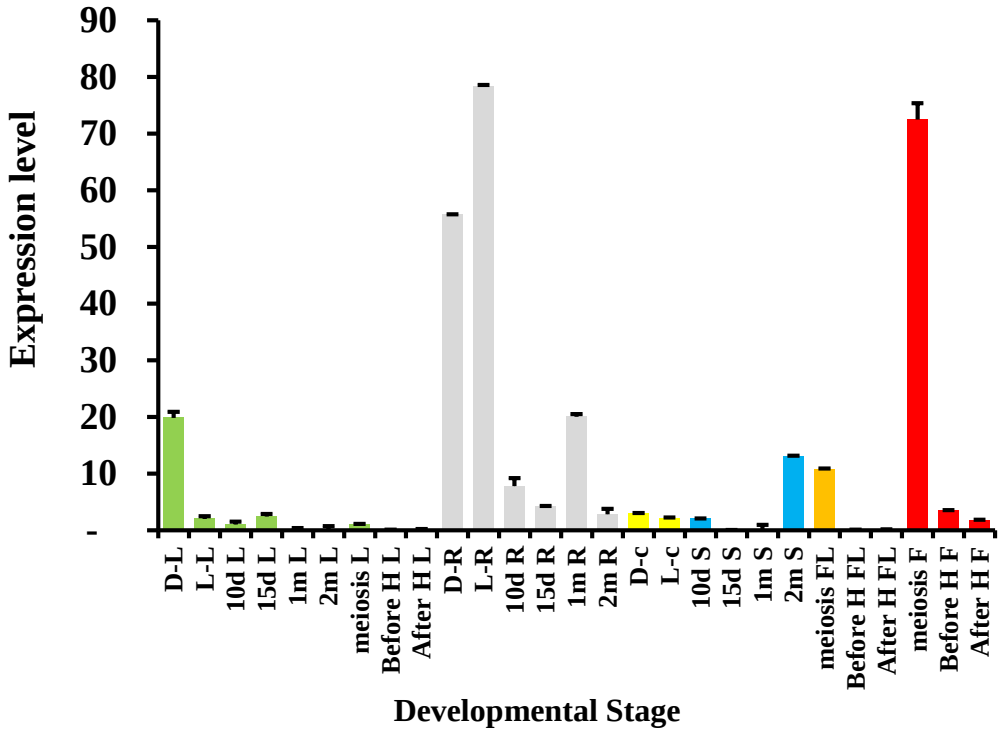
a.


400
300
200
100
-
Expression level
Leaf
NT
2
4
6
2
4
6
2
4
6
2
4
6
Drought
High Salinity
ABA
Low temperature
Time(h)

b.


400
300
200
100
-
Expression level
Root
NT
2
4
6
2
4
6
2
4
6
2
4
6
Drought
High Salinity
ABA
Low temperature
Time(h)

c.


Expression level
90
80
70
60
50
40
30
20
10
-
D-L
L-L
10d L
15d L
1m L
2m L
meiosis L
Before H L
After H L
D-R
L-R
10d R
15d R
1m R
2m R
D-c
L-c
10d S
15d S
1m S
2m S
meiosis FL
Before H FL
After H FL
meiosis F
Before H F
After H F
Developmental Stage

d.

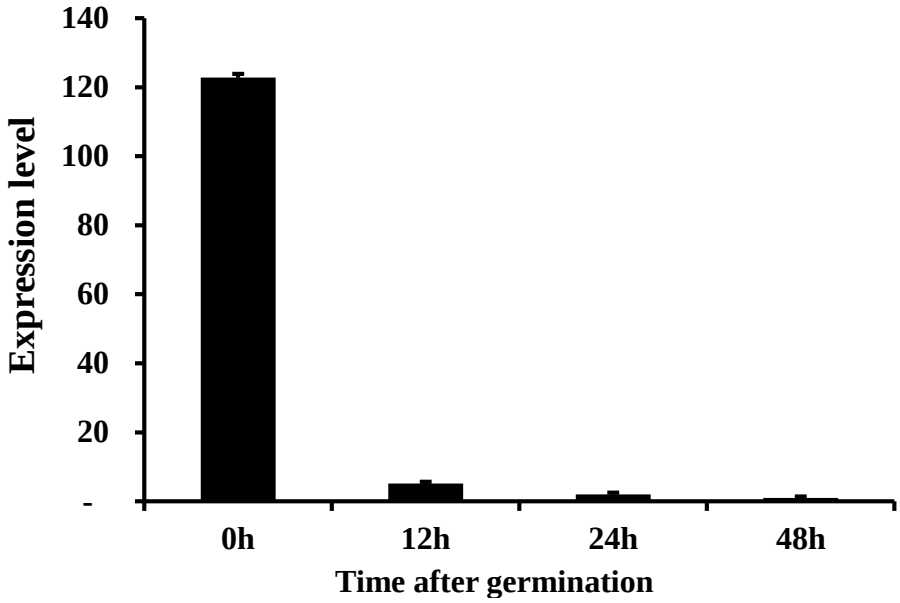

Expression level
140
120
100
80
60
40
20
-
0h
12h
24h
48h
Time after germination

Fig. 3 Enhanced drought stress tolerance visual tests of *PGD1:OsTZF8* and *RCc3:OsTZF8*

a. The phenotype of transgenic plants during drought stress for 3days. Four independent lines of *PGD1:OsTZF8* plants and NT Nakdong controls were grown for 5 weeks, first grown in Ms medium and transferred to soil after 1 week and then grown for another 4 weeks(n=30). The plants were exposed 4 d of drought stress, and followed by 5d of re-watering

b. Expression level of *PGD1:OsTZF8* plants, Shoot and root were checked separately.

c. Soil-moisture contents for drought stress treatment measured using Moisture Content BF 202, Japan E. of *PGD1:OsTZF8* plants.

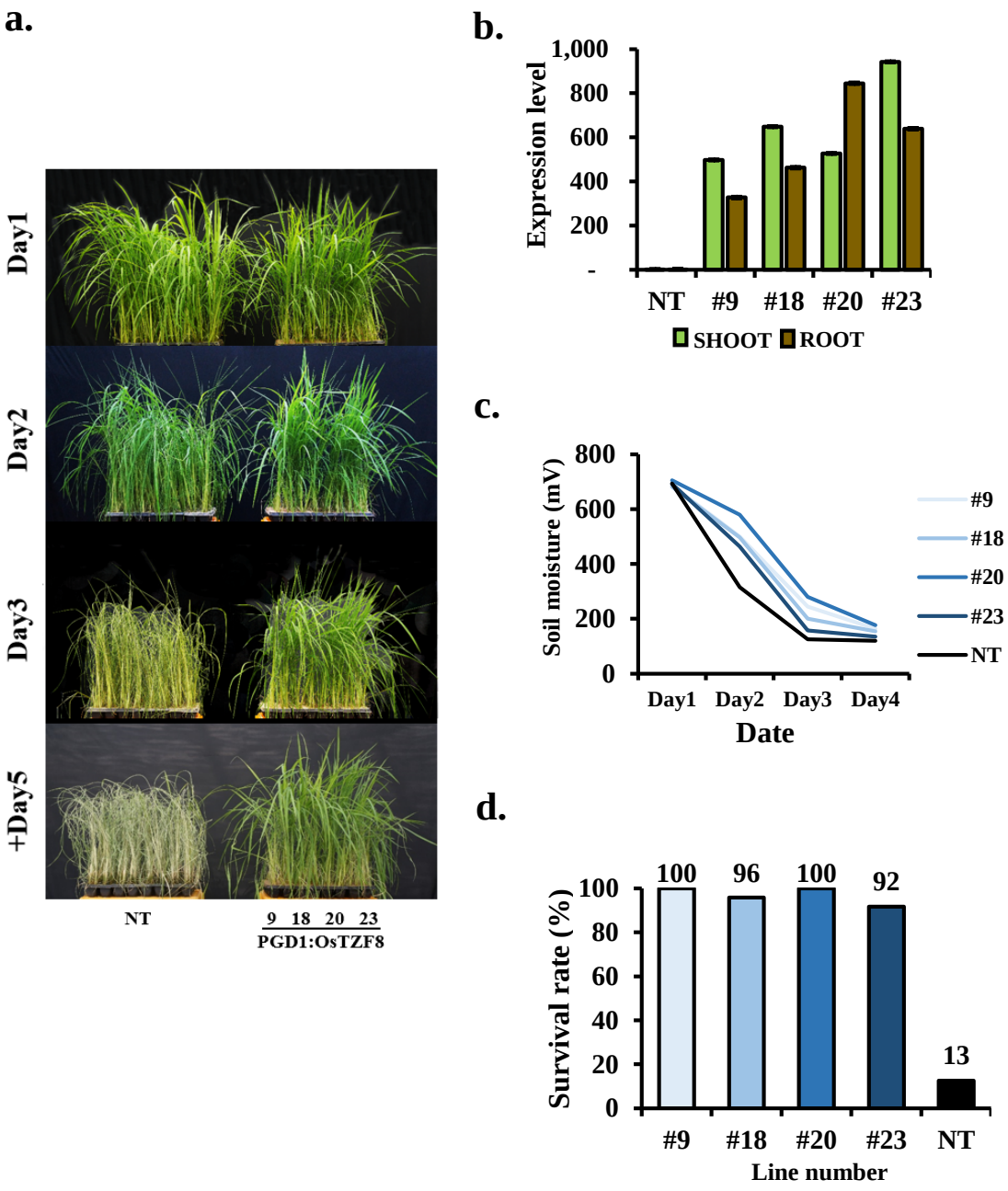
d. Survival rate for drought stress after 5 days of re-watering. Number of surviving plants in both the *PGD1:OsTZF8* transgenic and NT plants were counted and calculated by percentage (n=30).

e. The phenotype of transgenic plants during drought stress for 3days. Four independent lines of *RCc3:OsTZF8* plants and NT Illmi controls were grown for 5 weeks, first grown in Ms medium and transferred to soil after 1 week and then grown for another 4 weeks(n=30). The plants were exposed 3 d of drought stress, and followed by 5d of re-watering

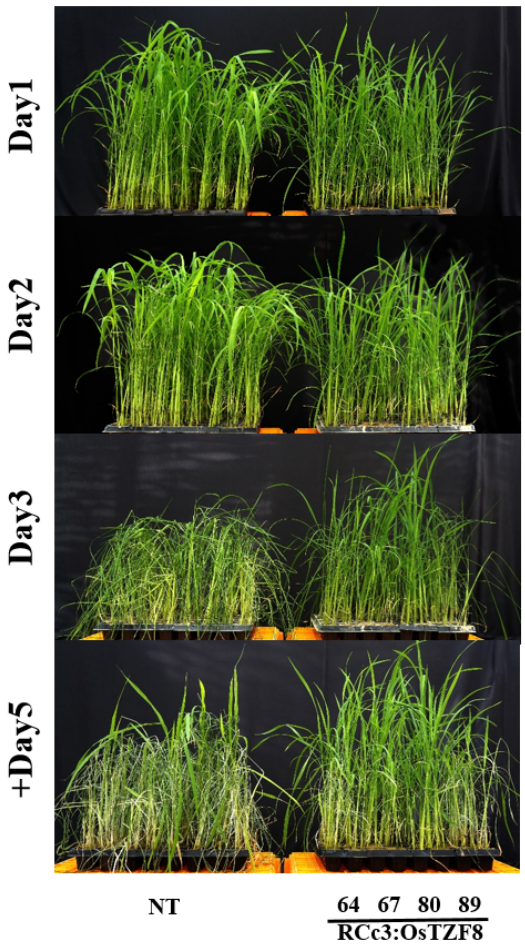
f. Expression level of *RCc3:OsTZF8* plants. Due to root specific promoter, the shoot expression level is relatively low, therefore hardly seen in the graph.

g. Soil-moisture contents for drought stress treatment measured using Moisture Content BF 202, Japan E. of *RCc3:OsTZF8* plants.

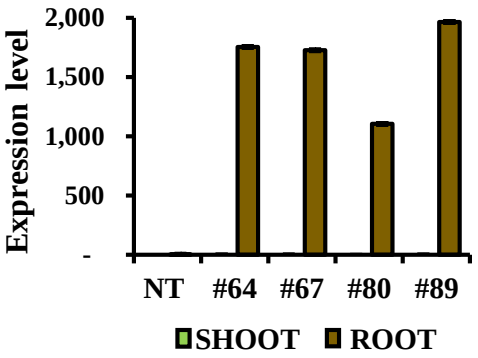
h. Survival rate for drought stress after 5 days of re-watering. Number of survived plants in both the *RCc3:OsTZF8* transgenic and NT plants were counted and calculated by percentage(n=30).


a.
Day1
Day2
Day3
+Day5
NT
9 18 20 23
PGD1:OsTZF8
b.
Expression level
1,000
800
600
400
200
-
NT #9 #18 #20 #23
SHOOT ROOT
c.
Soil moisture (mV)
800
600
400
200
0
Day1 Day2 Day3 Day4
Date
#9
#18
#20
#23
NT
d.
Survival rate (%)
100
80
60
40
20
0
100 96 100 92 13
#9 #18 #20 #23 NT
Line number

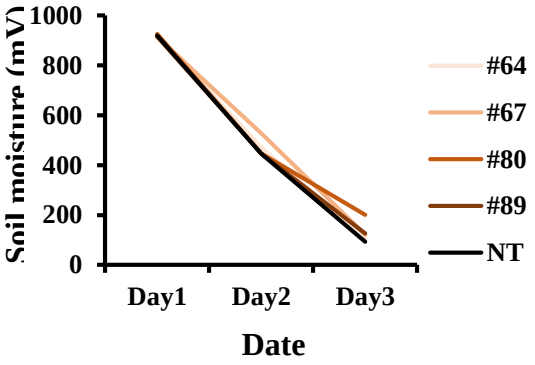
e.


Day1
Day2
Day3
+Day5
NT
64 67 80 89
RCc3:OsTZF8

f.


Expression level
2,000
1,500
1,000
500
-
NT #64 #67 #80 #89
SHOOT ROOT

g.


Soil moisture (mV)
1000
800
600
400
200
0
Day1 Day2 Day3
Date
#64
#67
#80
#89
NT

h.

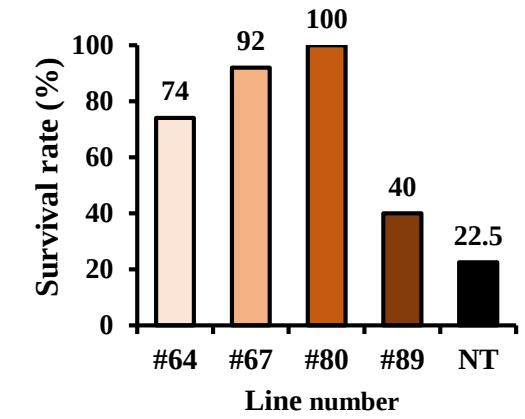
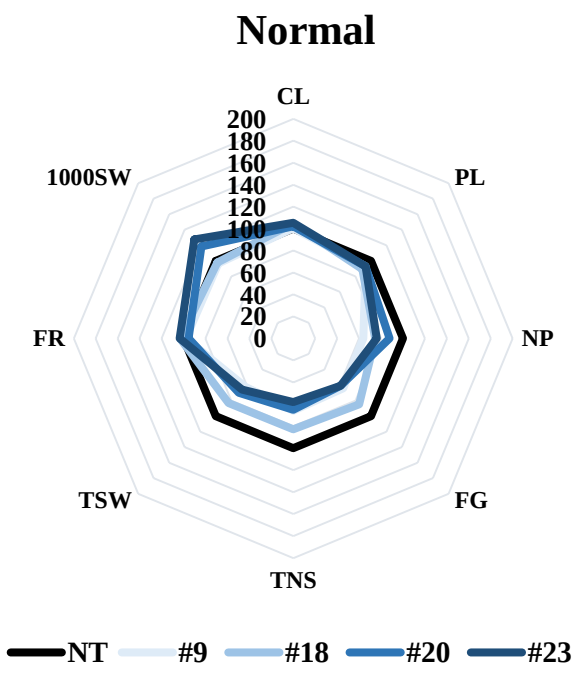

Survival rate (%)
100
80
60
40
20
0
74
92
100
40
22.5
#64 #67 #80 #89 NT
Line number

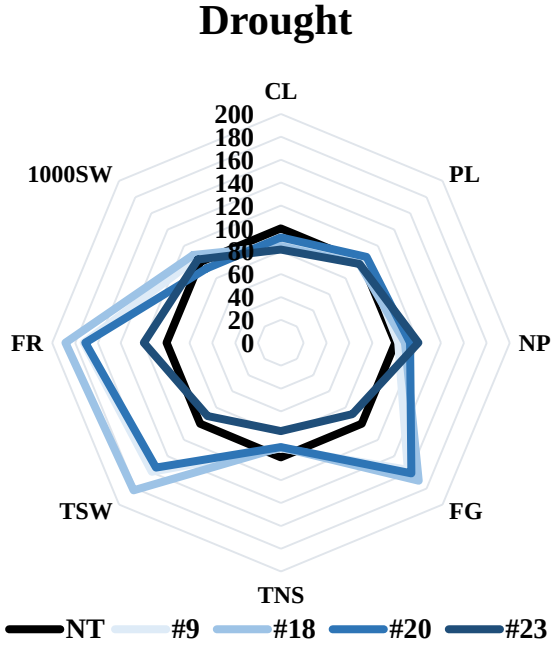
Fig. 4 Agronomic trait of Overexpression transgenic plant of *OsTZF8* and non-transgenic plant grown under normal and drought condition in the field.

Four independent *PGD1:OsTZF8* and *RCc3:OsTZF8* Plants were grown in the field for 3 months and under normal condition and drought condition. Each data point is the mean value (n=15) with the NT plants (n=18) showing the respective percentage considering the data of NT as 100% as a reference.CL, Colum length; PL, Panicle length; NP, Number of panicles; FG, Number of Filled Grain; NSP, Number of spikelet per panicle; TNS, Total number of seeds; FR, Filling rate; TSW, Total seed weight; 1000SW, 1000 Seed weight

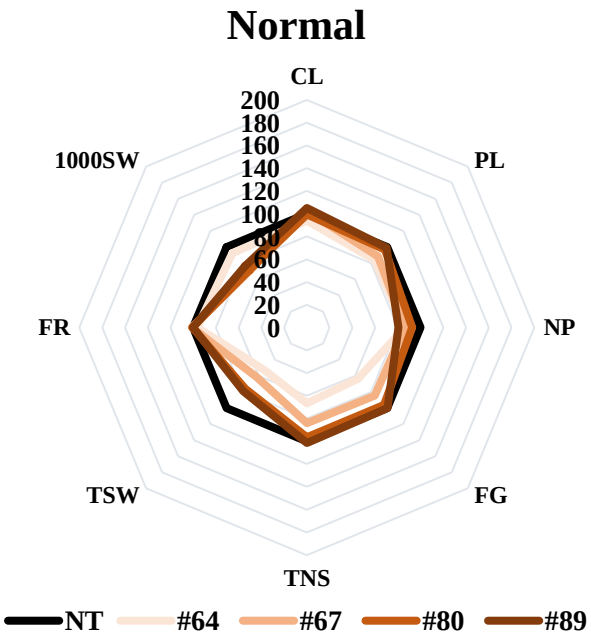
a.


Normal
CL
PL
NP
FG
TNS
TSW
FR
1000SW
200
180
160
140
120
100
80
60
40
20
0
NT
#9
#18
#20
#23

b.


Drought
CL
PL
NP
FG
TNS
TSW
FR
1000SW
200
180
160
140
120
100
80
60
40
20
0
NT
#9
#18
#20
#23

c.


Normal
CL
PL
NP
FG
TNS
TSW
FR
1000SW
200
180
160
140
120
100
80
60
40
20
0
NT
#64
#67
#80
#89

d.

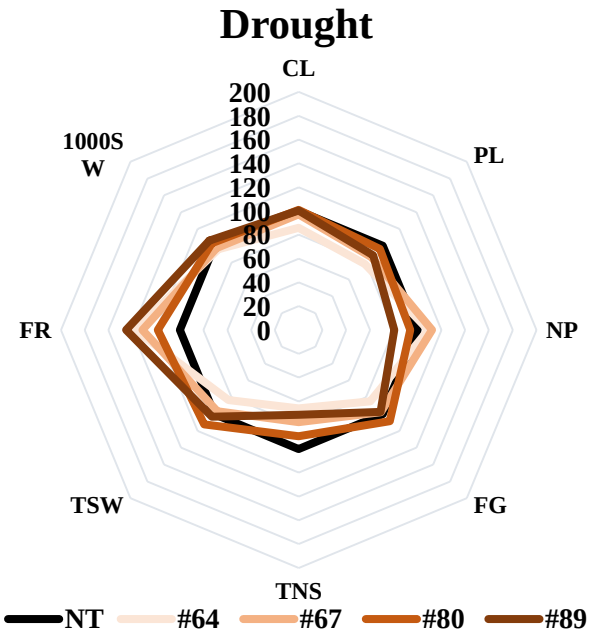

Drought
CL
PL
NP
FG
TNS
TSW
FR
1000S
W
200
180
160
140
120
100
80
60
40
20
0
NT
#64
#67
#80
#89

Fig. 5 Phenotypic characterization of grains in NT and *PGD1:OsTZF8* transgenic plant

Grain with rice bran (a) and without rice bran (b) of four independent *PGD1:OsTZF8* lines. 10 seeds were laid in a row beside with each other in order to observe the exact size change in both with and without rice bran. The vector of *PGD1:OsTZF8* contains *GFP* with stress inducible promoter *Wsi18:GFP* which show green fluorescence in stress condition. Since grain is in a dehydrated state, OX line grains without rice bran show green color

a.

b.

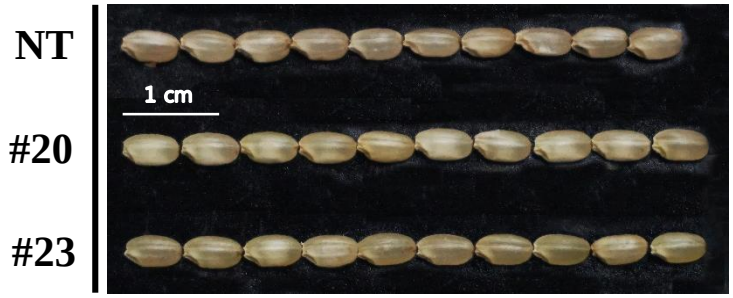
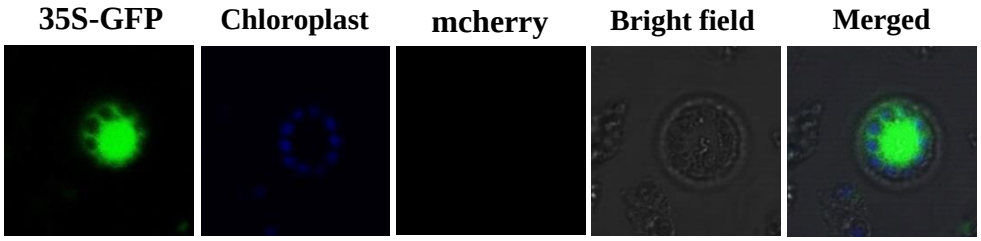


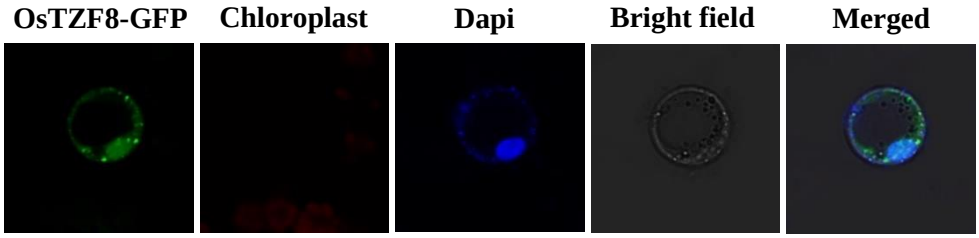
Fig. 6 Subcellular localization of *OsTZF8*

Schematic structure was taken by confocal microscope. The vector with 35S promoter and GFP tagged at the end of the gene was observed using visible protoplast. The sample except control sample and dapi stained sample, was given 0-3 hours of heat stress to observe the pattern of cytoplasmic foci made on stress condition. Chloroplast (auto fluorescence) is shown in blue color in Fig 6a, c, d not to be confused with RFP (mcherry)

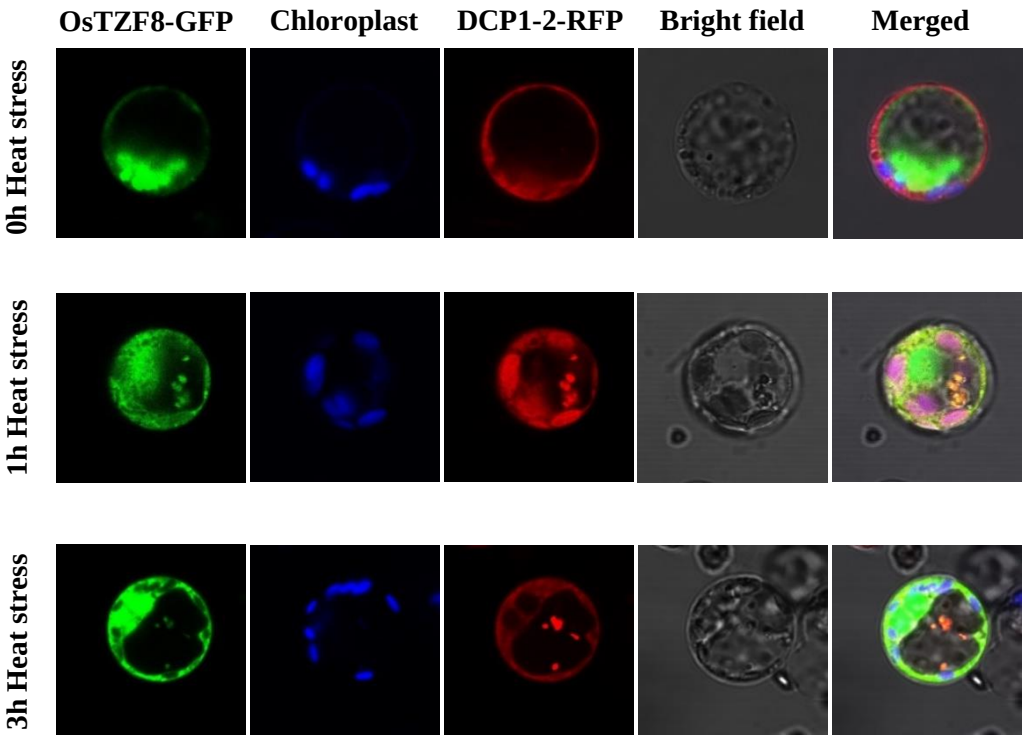
a.


35S-GFP
Chloroplast
mcherry
Bright field
Merged

b.


OsTZF8-GFP
Chloroplast
Dapi
Bright field
Merged

c.


OsTZF8-GFP
Chloroplast
DCP1-2-RFP
Bright field
Merged
0h Heat stress
1h Heat stress
3h Heat stress

d.

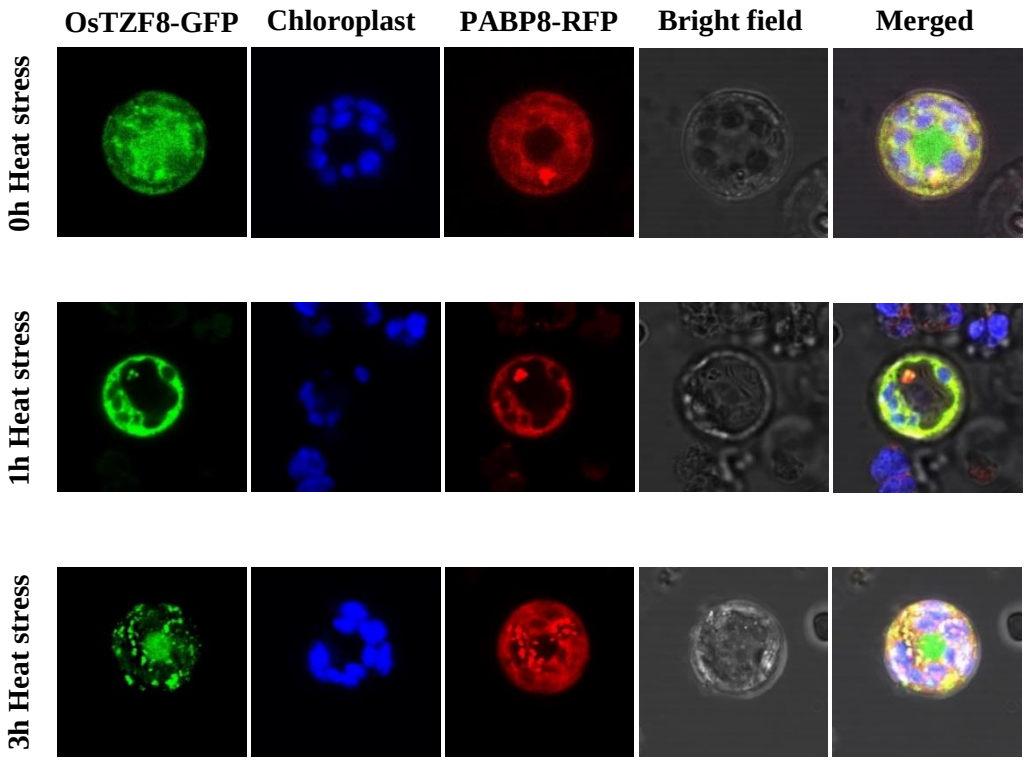
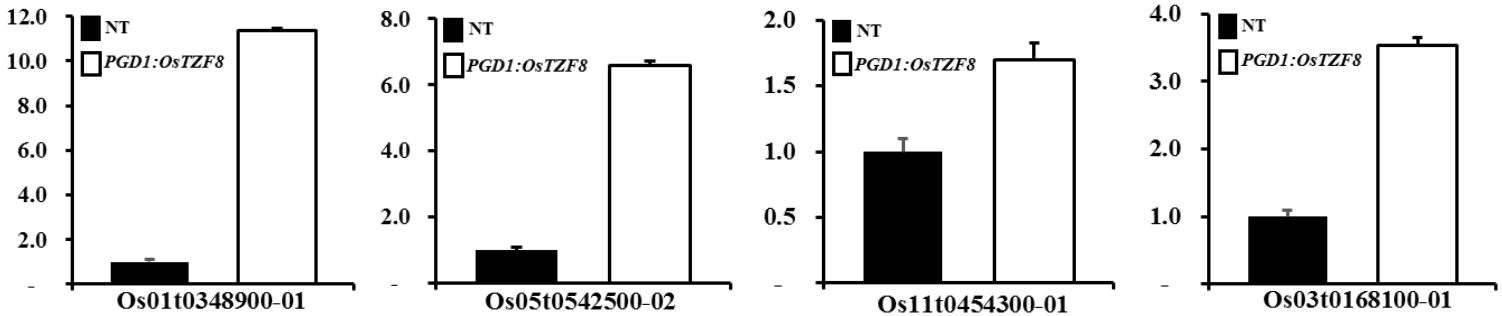

OsTZF8-GFP
Chloroplast
PABP8-RFP
Bright field
Merged
0h Heat stress
1h Heat stress
3h Heat stress

Fig. 7 Validation of *OsTZF8* target genes

7 genes out of 22 genes from RNA seq is narrowed as direct target gene after validated by qRT-PCR. Os01g0348900 (Salt-induced protein), Os05g0542500 (LEA-like protein), Os11g0454200 (Dehydrin RAB 16B), Os03g0168100 (Late embryogenesis abundant protein repeat containing protein), Os08g0189100 (Germin-like protein 8-2, Disease resistance), Os08g0189400 (Germin-like protein precursor.) Os12g0154800 (RmlC-like jelly roll fold domain containing protein)


12.0
10.0
8.0
6.0
4.0
2.0
-
NT
PGD1:OsTZF8
Os01t0348900-01
8.0
6.0
4.0
2.0
-
NT
PGD1:OsTZF8
Os05t0542500-02
2.0
1.5
1.0
0.5
-
NT
PGD1:OsTZF8
Os11t0454300-01
4.0
3.0
2.0
1.0
-
NT
PGD1:OsTZF8
Os03t0168100-01

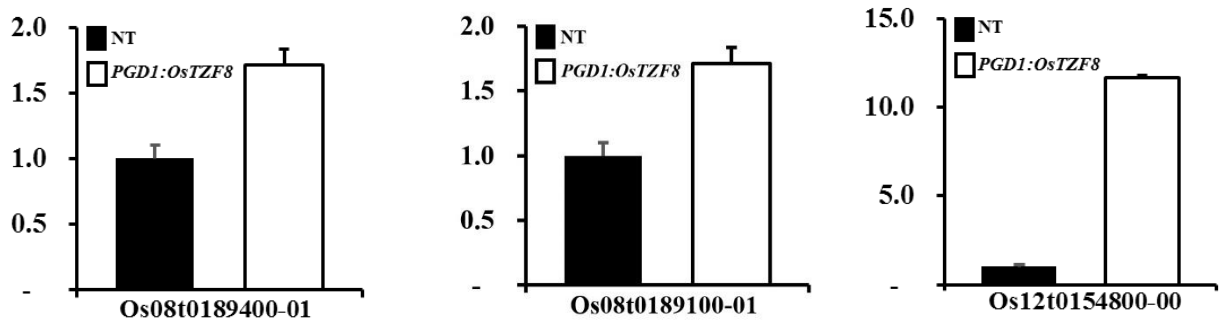

2.0
1.5
1.0
0.5
-
NT
PGD1:OsTZF8
Os08t0189400-01
2.0
1.5
1.0
0.5
-
NT
PGD1:OsTZF8
Os08t0189100-01
15.0
10.0
5.0
-
NT
PGD1:OsTZF8
Os12t0154800-00

Table 1. Candidates of *OsTZF8* target genes

	No.	Transcript_ID	Description	OsTZF8/ NT fold change	GO term	Log2 Ratio(Drought) 1d	2d	3d
	1	Os01t0348900-01	SalT gene product (Salt-induced protein).	3.8		1.1	6.2	8.6
LEA	2	Os05t0542500-02	LEA-like protein.	11.7	-	1.3	7.3	7.8
LEA	3	Os11t0454300-01	Similar to Water-stress inducible protein RAB21.	8.5	response to stress, response to water	1.2	6.8	8.6
LEA	4	Os11t0454200-01	Dehydrin RAB 16B.	3.9	response to stress, response to water	1.5	7.3	8.6
LEA	5	Os01t0705200-01	Late embryogenesis abundant protein repeat containing protein.	3.2	-	1.4	7.8	8.1
LEA	6	Os03t0168100-01	Late embryogenesis abundant protein repeat containing protein.	3.1	-	1.2	7.1	9.0
LEA	7	Os04t0589800-01	Late embryogenesis abundant (LEA) group 1 family protein.	2.9	embryo development	1.1	7.1	8.3
LEA	8	Os11t0451700-00	Similar to Dehydrin DHN1 (M3) (RAB-17 protein).	2.5	response to stress, response to water	1.1	7.4	8.2
Germin-like	9	Os12t0154900-00	Germin-like protein 12-3, Disease resistance	3.2	apoplast			
Germin-like	10	Os12t0154700-01	Germin-like protein 12-1, Disease resistance	3.1	apoplast			
Germin-like	11	Os08t0189700-01	Germin-like protein 8-8, Disease resistance	2.4	apoplast		2.6	0.3
Germin-like	12	Os12t0155000-00	Similar to Germin-like protein subfamily 1 member 8.	2.3				
Germin-like	13	Os08t0189100-01	Germin-like protein 8-2, Disease resistance	2.3	apoplast, manganese, metal-binding	-0.3	0.3	2.8
Germin-like	14	Os12t0154800-00	RmlC-like jelly roll fold domain containing protein.	2.2	apoplast, manganese, metal-binding			
Germin-like	15	Os08t0189400-00	Germin-like protein precursor.	2.1				
PR	16	Os07t0127700-01	Similar to Pathogenesis-related protein class 1.	4.9	-			
PR	17	Os11t0591800-01	Barwin domain containing protein.	3.8	defense response to bacterium, defense response to fungus			
PR	18	Os03t0661600-01	Similar to Alpha-amylase/trypsin inhibitor (Antifungal protein).	3.8	-	0.3	2.7	5.2
PR	19	Os03t0663400-02	Similar to Thaumatin-like protein.	2.7	-	-0.4	1.3	3.2
PR	20	Os07t0127600-01	Allergen V5/Tpx-1 related family protein.	2.7	-			
PR	21	Os11t0592000-01	Similar to Barwin.	2.7	defense response to bacterium, defense response to fungus	-0.3	1.8	3.5
PR	22	Os12t0437800-01	Similar to MPI.	2.6	response to wounding	1.6	6.1	6.8

Table 2. Primer information

Primer name	Sequence
qRT-PCR	
OsTZF8-QRT-F	GGACATGAAGCAGATTGTCCT
OsTZF8-QRT-R	CCCATCCCAGTTCCGGAGC
pHBT-OsTZF8-GFP	
OsTZF8-GFP tag-F	CGGGATCCATGGCATACGAGACGT
OsTZF8-GFP tag-R	TATAGCGGCCGCCCATCAACAGGT
OsDCP1-2 (Os12g0156400)	
DCP1-2-full-F	GGGCCTCGGTTTGCTCAGAT
DCP1-2-full-R	ACTCTCGTCAAGGCCAGACA
DCP1-2-RFP_B-F	TTGCTCCGTGGATCCATGCGGCCGCCGGCGCCGGC
DCP1-2-RFP_N-R	AAAGCGGCCGCAAATCGCATGTGCATTCTGTAACT
OsPABP8 (Os09g0115400)	
OsPABP8-orf-F	GCCGCAGTTGCAGTTGCAGT
OsPABP8-orf-R	CCAATCCACGCGCATTGCCT
PABP8-RFP_B-F	TTGCTCCGTGGATCCATGGCGGCGGCGGCGGCGCAG
PABP8-RFP_N-R	AAAGCGGCCGCAAATGGAGGAAACGACGCCGTCAAT
OsUbiquitin1 (Os06g0681400)	
Ubi-F	ATGGAGCTGCTGCTGTTCTA
Ubi-R	TTCTTCCATGCTGCTCTACC
SalT1(Os01g0348900)	
OsSalT1-F	GGAACGCTTATCGACGCAAT
OsSalT1-R	GGTGCAACACGTACACAGAC
LEA-like(Os05g0542500)	
OsLEA3-1-F	CCACACCGAGGAGAAGGCG
OsLEA3-1-R	GTCGCCTCCTTGGTATCCTGC
Dehydrin RAB 16B(Os11g0454200)	
OsRAB16B-F	GTTCCAGCCGATGAGGGAG
OsRAB16B-R	CATGGCATGCTGCTGCTC
LEA repeat(Os03g0168100)	
OsLEA16-F	GTGGCGACGAAGGCGGAG
OsLEA16-R	CTCCTCCTCGTCGACGTCG
Germin-like 8-2(Os08g0189100)	
OsGLP8-2-F	TCGCTGATCTGAATTCGCCA
OsGLP8-2-R	CTTATCGAGCATGGCTGCCT
Germin-like precursor(Os08g0189400)	
OsGLP8-5-F	ACGGTGCTTGAGGGAACATT
OsGLP8-5-R	CAGCAGGCTGATGTGGGTTA
(RmlC-like jelly roll fold(Os12g0154800)	
OsGLP12-2-F	TGATAAGCCAGCAGTTGCCA
OsGLP12-2-R	TTGGAGCCAATCCACAGCTT

Table 3.Agronomic traits for PGD1:OsTZF8 and RCc3:TZF8 in normal condition

Normal		Culm length	Panicle length	Number of Panicle	Filled grain	Unfilled grain	Total number of seed	Total seed weight	Filling rate	1000 seed weight
NT	Average	69.00 ± 3.92	21.3 ± 1.7	21.7 ± 4.07	1946.3 ± 431	245.67 ± 76.7	2192 ± 466	49.3 ± 466	88.6 ± 3.66	25.4 ± 0.68
#9	Average	69.17 ± 4.22	19.8 ± 1.21	13.8 ± 3.02	1252.7 ± 333	192.83 ± 107	1445.5 ± 370	30.6 ± 7.97	86.8 ± 5.76	24.5 ± 1.28
	%Δ	0.25	-7.03	-36.2	-35.639	-21.509	-34.056	-38	-2.06	-3.58
	p-value	0.265	0.395	0.136	0.317	0.014	0.153	0.249	0.187	0.013
#18	Average	72.3 ± 0.75	19 ± 0.82	16.2 ± 3.8	1653 ± 353	159.17 ± 78.3	1812.17 ± 407	41.1 ± 8.92	91.5 ± 2.52	24.9 ± 0.72
	%Δ	4.83	-10.9	-25.4	-15.071	-35.21	-17.328	-16.6	3.19	-2.2
	p-value	0.207	0.229	0.383	0.496	0.592	0.094	0.076	0.062	0.394
#20	Average	70.5 ± 2.36	19.8 ± 1.46	19 ± 8	1190.2 ± 397	242.33 ± 168	1423.5 ± 539	34.5 ± 13.2	84.4 ± 5.64	30.1 ± 8.37
	%Δ	2.17	-7.03	-12.3	-38.851	-1.3595	-35.059	-29.9	-4.75	18.5
	p-value	0.514	0.356	0.471	0.135	0.274	0.245	0.186	0.329	0.076
#23	Average	72.7 ± 2.21	19.8 ± 1.07	16.5 ± 5.38	1185.2 ± 688	87 ± 31.9	1272.17 ± 716	32.5 ± 12.4	91.9 ± 2.71	32.5 ± 9.16
	%Δ	5.32	-7.03	-23.9	-39.107	-64.587	-41.963	-34	3.63	27.7
	p-value	0.731	0.085	0.173	0.425	0.168	0.016	0.253	0.149	0.027
NT	Average	69.67 ± 1.37	21.83 ± 1.34	18.00 ± 3.21	1511.67 ± 344	165.17 ± 58.5	1676.83 ± 298	49.20 ± 12.1	89.15 ± 6.38	33.48 ± 8.17
#64	Average	65.5 ± 4.27	18.3 ± 0.75	15.5 ± 2.29	962.17 ± 418	155 ± 63.7	1117.17 ± 478	25.9 ± 4.18	85.9 ± 1.44	31 ± 9.39
	%Δ	-5.99	-16	-13.9	-36.351	-6.1573	-33.376	-47.3	-3.67	-7.47
	p-value	0.207	0.114	0.165	0.393	0.461	0.241	0.334	0.167	0.29
#67	Average	71.5 ± 2.34	19.3 ± 0.47	15.5 ± 2.29	1278 ± 178	130.67 ± 58.3	1408.67 ± 143	30.5 ± 5.04	90.4 ± 5.09	23.8 ± 0.84
	%Δ	2.63	-11.5	-13.9	-15.458	-20.888	-15.992	-38	1.36	-29.1
	p-value	0.517	0.016	0.703	0.281	0.149	0.673	0.413	0.034	0.184
#80	Average	69.2 ± 2.97	21.2 ± 0.9	16.7 ± 2.75	1451.3 ± 250	163.5 ± 31.3	1614.83 ± 267	37.5 ± 6.11	89.8 ± 1.85	23.9 ± 0.58
	%Δ	-0.72	-3.02	-7.39	-3.9916	-1.0111	-3.6975	-23.8	0.73	-28.6
	p-value	0.122	0.367	0.162	0.219	0.345	0.034	0.226	0.177	0.009
#89	Average	73.2 ± 2.11	21.7 ± 0.94	14.5 ± 1.61	1516.3 ± 170	185.83 ± 45.4	1702.17 ± 196	38.9 ± 4.3	89.2 ± 2.09	25.7 ± 0.54
	%Δ	5.02	-0.73	-19.4	0.3083	12.5083	1.51118	-21	0	-23.4
	p-value	0.473	0.101	0.426	0.318	0.352	0.072	0.623	0.247	0.239

Table 4.Agronomic traits for PGD1:OsTZF8 and RCc3:TZF8 in drought condition

Drought		Culm length	Panicle length	Number of Panicle	Filled grain	Unfilled grain	Total number of seed	Total seed weight	Filling ratae	1000 seed weight
NT	Average	78.88 ± 5.55	17.765 ± 1.628	19 ± 4.39	637.41 ± 327	1215.529 ± 386	1852.941 ± 557	13.67 ± 7.67	34.38 ± 11.78	21.01 ± 2.34
#9	Average	68.39 ± 4.37	18.5 ± 1.803	19.5 ± 2.63	1000.2 ± 213	711.2222 ± 284	1711.444 ± 218	21.8 ± 5	59.25 ± 13.2	21.89 ± 2.26
	%Δ	-13.3	4.1391	2.632	56.919	-41.4887	-7.63633	59.44	72.33	4.186
	p-value	0.007	0.148	0.018	0.312	0.241	0.041	0.072	0.256	0.012
#18	Average	66.78 ± 3.561	17.278 ± 1.951	20.61 ± 7.53	1087 ± 473	609.3889 ± 277	1696.389 ± 563	24.91 ± 9.37	64.79 ± 13.4	22.85 ± 2.2
	%Δ	-15.3	-2.741	8.48	70.533	-49.8664	-8.44885	82.19	88.45	8.72
	p-value	0.219	0.152	0.096	0.422	0.239	0.188	0.095	0.111	0.025
#20	Average	72.61 ± 3.561	18.833 ± 1.951	21.44 ± 7.53	1029 ± 473	661.5 ± 277	1690.5 ± 563	21.11 ± 9.37	58.81 ± 13.4	19.32 ± 2.2
	%Δ	-7.95	6.0155	12.87	61.434	-45.5793	-8.76667	54.41	71.06	-8.06
	p-value	0.145	0.275	0.046	0.085	0.410	0.381	0.074	0.317	0.104
#23	Average	64.39 ± 2.965	17.278 ± 1.483	22.83 ± 3.32	563.06 ± 400	869.1667 ± 247	1432.222 ± 213	12.39 ± 4.74	41.16 ± 18.4	21.6 ± 5.44
	%Δ	-18.4	-2.741	20.18	-11.665	-28.4948	-22.7055	-9.35	19.71	2.804
	p-value	0.261	0.343	0.168	0.133	0.024	0.008	0.438	0.165	0.273
NT	Average	67.89 ± 2.885	19.89 ± 2.131	17.78 ± 3.52	602.00 ± 342	1006.44 ± 374	1608.44 ± 390	13.01 ± 6.86	37.72 ± 19.7	22.05 ± 1.72
#64	Average	58.38 ± 2.666	15.625 ± 0.599	19.38 ± 3.37	511.69 ± 166	546.75 ± 313	1058.438 ± 310	10.83 ± 3.76	51.89 ± 18.2	21.12 ± 1.75
	%Δ	-14	-21.439	8.984	-15.002	-45.6751	-34.195	-16.8	37.59	-4.21
	p-value	0.002	0.144	0.396	0.297	0.041	0.106	0.483	0.004	0.008
#67	Average	66.06 ± 5.027	17.222 ± 1.583	19.94 ± 3.41	589.22 ± 194	656.1111 ± 358	1245.333 ± 300	12.58 ± 4.22	49.56 ± 17.6	21.5 ± 1.62
	%Δ	-2.7	-13.408	12.19	-2.1226	-34.809	-22.5753	-3.29	31.41	-2.5
	p-value	0.154	0.299	0.164	0.025	0.342	0.124	0.166	0.013	0.057
#80	Average	68.59 ± 4.815	19.176 ± 1.79	16.71 ± 3.34	652.24 ± 323	786.5882 ± 347	1438.824 ± 361	14.59 ± 7.86	44.69 ± 21.2	22.76 ± 3.91
	%Δ	1.03	-3.582	-6.03	8.3447	-21.8448	-10.5456	12.17	18.49	3.207
	p-value	0.275	0.228	0.437	0.321	0.149	0.003	0.226	0.129	0.159
#89	Average	68.12 ± 10.03	17.647 ± 2.195	14.29 ± 6.39	585.35 ± 376	563.3529 ± 352	1148.706 ± 589	13.42 ± 8.88	54.72 ± 18.4	23.46 ± 4.47
	%Δ	0.337	-11.272	-19.6	-2.7653	-44.0254	-28.5828	3.17	45.07	6.389
	p-value	0.352	0.002	0.265	0.061	0.042	0.189	0.247	0.496	0.274

DISCUSSION

Some members of the Tandem Zinc finger family are already known to have a response to abiotic stresses (Sun et al., 2007, Asad et al., 2013). In this study, another member *OsTZF8* is introduced as stress induced gene. The alignment of these TZF genes reveals that members with similar protein sequence tends to share similar property linked to abiotic stress. Transgenic plants which are overexpressing *OsTZF8*, *PGD1:OsTZF8* and *RCc3:OsTZF8* demonstrate higher tolerance in drought stress. The change in plant size in agronomic trait of *PGD1:OsTZF8* may have driven from an energy shift from growth to defense, indeed increasing the tolerance yet shrink the overall size of the plant. This may connect to the fact that the agronomic trait of *RCc3:OsTZF8* showed less contrast between transgenic and non- transgenic when it shows less tolerance to drought than *PGD1:OsTZF8*.

Expression level in different development stage implies that higher expression was screened at some root in the vegetative stage (Fig 2c). This can be connected to the experiment of expression level in four different abiotic stresses showing the root show higher response to ABA which is a stress hormone. (Fig 2b). Nevertheless, as the expression during reproductive stage is significantly higher, *OsTZF8* seems to play more important role in above ground part of the plant rather than underground part. By the graph of expression level at an early stage of germination (Fig 2d) showing that the expression of *OsTZF8* sharply drops after germination and decrease slowly, *OsTZF8* can be considered to participate not in germination but at the embryo formation. In fact, the result that the weight of the seed increased in *PGD1:OsTZF8*, can lead to a hypothesis that *OsTZF8* functions in

seed metabolism. As some of the members in TZF family are already studied to participate in embryogenesis (Li and Thomas, 1998), there is a high chance of *OsTZF8* to play a role in embryogenesis as well. The best approach to prove this fact is to design an RNAi or knockout transgenic to check whether there is an influence in embryogenesis stage.

In RNA-sequencing data, many of late embryogenesis abundant proteins (LEA) tend to be up-regulated by *OsTZF8* (Table 1). LEA gene is known to express at late embryogenesis stage to protect DNA, RNA and proteins and preserve in embryo in order to keep it until the next generation (Leon et al., 1981). During this stage, plant exposes itself to massive osmotic stress to cease all other functions and concentrate in embryo formation. If many LEA genes are directly regulated by *OsTZF8* then it is also a key factor in regulating the embryo formation and stress defense (Shao et al., 2005). As other defense genes like Germin-like protein (GLP) and Pathogenesis related (PR) are also candidate of *OsTZF8*, there is a high chance that *OsTZF8* protects plant from various stresses. The validation result using qRT-PCR, persuades the fact that *OsTZF8* may also regulate salt stress response as the Os01g0348900 (Salt-induced protein) is one of the direct target genes. Similar to previous paper about CCCH family zinc finger protein regulating salt stress (Sun et al., 2007), *OsTZF8* may also play important roles in modulating salt stress tolerance. Moreover, some LEA genes and germin-like genes seems to have direct control from *OsTZF8* indicating the possibility of both embryo formation and stress defense.

One of the result that draws attention is the co-localization of *OsTZF8* with both processing bodies (PB) and stress granules (SG) in protoplast. As both organs are known to participate in RNA turnover, one of the reasons, *OsTZF8* provides

drought tolerance may linked to RNA protection in stress condition. However, with our results, there is no specific clue whether *OsTZF8* functions as a transcriptional regulator or has a role in RNA metabolism. Therefore, this result in subcellular localization leaves us a direction for further study that can be continued afterward. In further experiments, methods such as a transactivation assay to verify whether *OsTZF8* is transcription factor or a DNA or RNA binding protein is the most crucial step to follow.

REFERENCES

Aldemita RR, Reano IM, Solis RO, Hautea RA (2015) Trends in global approvals of biotech crops (1992–2014). *GM Crops Food* **6**, 150–166

Anderson P, Kedersha N (2008) Stress granules: the Tao of RNA triage.

Trends Biochem Sci **33,** 141–150

Chung PJ, Jung H, Jeong DH, Ha SH, Choi YD, Kim JK (2016) Transcriptome profiling of drought responsive noncoding RNAs and their target genes in rice. *BMC Genomics*. **17**, 563

Kamthan A, Chaudhuri A, Kamthan M, Datta A (2016) Genetically modified (GM) crops: milestones and new advances in crop improvement. *Theor Appl Genet* **129**, 1639–1955

Kim DH, Yamaguchi S, Lim S, Oh E, Park J, Hanada A, Kamiya Y, Choi G (2008) SOMNUS, a CCCH-type zinc finger protein in Arabidopsis, negatively regulates light-dependent seed germination downstream of PIL5. *Plant Cell* **20,** 1260–1277

Iwasaki S, Takeda A, Motose H, Watanabe Y (2007) Characterization of Arabidopsis decapping proteins AtDCP1 and AtDCP2, which are essential for post-embryonic development. *FEBS Lett.* **581**, 2455–2459

Jan A, Maruyama K, Todaka D, Kidokoro S, Abo M, Yoshimura E, Kazuo S, Kazuo N, and Kazuko Y-S (2013) OsTZF1, a CCCH-Tandem Zinc Finger Protein, Confers Delayed Senescence and Stress Tolerance in Rice by Regulating Stress-Related Genes. *Plant Physiology.* **161,** 1202–1216

Jung,H.,Lee,D.K., Choi,Y.D.,and Kim,J-K.(2015).OsIAA6, a member of the rice Aux/IAA gene family, is involved in drought tolerance and tiller outgrowth. Plant Science. **236**, 304-12

Lee DK, Jung H, Jang G, Jeong JS, Kim YS, Ha SH, Choi YD, Kim JK (2016)

Overexpression of the OsERF71 transcription factor alters rice root structure and drought resistance. *Plant Physiology* **172**,575–588

Leon D III, Sally C. G and Glenn A. G (1981) Developmental Biochemistry of Cottonseed Embryogenesis and Germination: Changing Messenger Ribonucleic Acid Populations As Shown by in Vitro and in Vivo Protein Synthesis. *Biochemistry* **20**, 4162-4168

Li Z, Thomas TL (1998) PEI1, an embryo-specific zinc finger protein gene

required for heart-stage embryo formation in Arabidopsis. *Plant Cell* **10,** 383–398

Newbury SF, Mühlemann O, Stoecklin G (2006) Turnover in the Alps: an mRNA perspective. Workshops on mechanisms and regulation of mRNA turnover. *EMBO Re.p* **7**, 143–148

Oh SJ, Kim YS, Kwon CW, Park HK, Jeong JS, Kim JK (2009) Overexpression of the Transcription factor AP37 in Rice Improves Gran Yield under Drought Conditions. Plant physiology.**150**, 1368-1379

Pomeranz M, Lin PC, Finer J and Jang JC (2010) AtTZF gene family localizes to cytoplasmic foci. *Plant Signal & Behavior.* **5,** 190–192.

Shao HB, Liang ZS, Shao MA (2005) LEA proteins in higher plants: Structure, function, gene expression and regulation. *Colloids and Surfaces B: Biointerfaces* **45**, 131–135

Sun J, Jiang H, Xu Y, Li H,Wu X, Xie Q, Li C (2007) The CCCH-type zinc finger proteins AtSZF1 and AtSZF2 regulate salt stress responses in Arabidopsis. *Plant Cell Physiology* **48,** 1148–1158

Varshney RK, Bansal KC, Aggarwal PK, Datta SK, Craufurd PQ (2011) Agricultural biotechnology for crop improvement in a variable climate: hope or hype? *Trends Plant Sci* **16**, 363–371

Wang, D., Guo, Y., Wu, C., Yang, G., Li, Y. and Zheng, C. (2008) Genome-wide analysis of CCCH zinc finger family in Arabidopsis and rice. *BMC Genomics* **9**, 44

Weber C, Nover L, Fauth M (2008) Plant stress granules and mRNA processing

bodies are distinct from heat stress granules. *Plant J* **56**, 517–530

ABSTRACT IN KOREAN

CCCH –Tandem Zinc finger인 *OsTZF8*의 과발현 벼의 가뭄저항성 증가에 대한 연구

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Zinc finger 단백질은 중 하나인 **Tandem CCCH Zinc finger (TZFs)**는 진핵생물에서 유전자 발현의 전사 후 조절에 관여하는 것으로 알려져 있다. 그러나 가뭄 스트레스의 관련한 기능은 아직 잘 알려지지 않았다. 본 연구에서는 **TZF** 인 벼 유전자 ***OsTZF8*** 이 비 생물적 스트레스에 의해 유도되고 과발현 형질전환벼는 가뭄 저항성이 높다는 것을 확인하였다. 유전자 발현 분석 결과, ***OsTZF8*** 은 배아에서 특이적으로 발현되었으나 다른 조직에서는 발현되지 않았다. 포장 분석결과에서는 가뭄 상태에서 항시 과발현 형질전환벼가 야생 벼보다 수율이 높았고 벼의 크기가 커졌다. ***OsTZF8*** 은 핵에 뿐만 아니라, 스트레스 조건 하에서 **cytoplasmic foci** 을 형성함으로써 활성화되는 것으로 알려진 2 개의 메신저 리보 – 핵 단백질 복합체 **processing bodies** 및 **stress granules** 과 공동 – 위치한다. 요약하면, 이 결과는 ***OsTZF8*** 이 **processing bodies** 와 **stress granules** 에서 **RNA turnover** 에 참여함으로써 벼의 가뭄 스트레스를 조절한다는 것을 제시한다.

키워드: **Tandem CCCH zinc finger, 벼, 가뭄저항성, Processing bodies (PB), Stress granules (SG)**